

Multiple Paternity in *Littorina obtusata* (Gastropoda, Littorinidae) Revealed by Microsatellite Analyses

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Abstract. Parental identity for juvenile *Littorina obtusata* was determined from three egg masses by means of microsatellite DNA markers. Results confirm that the attendant adult female in each case was the dam of the offspring and that at least 4–6 males contributed to each brood. This correlates with our behavioral observations that indicated multiple copulations between the female and several males in each experimental aquarium. A significant number of offspring from each brood were sired by non-sampled males (males that had copulated with females before capture) whose sperm had been stored by the female. This is the first direct evidence of multiple paternity in the Littorinidae. Results are discussed in reference to current theories of sperm competition, male precedence, and cryptic female choice.

Introduction

Littorinids, as well as most other gastropods, fertilize internally. Sperm are stored temporarily in the bursa copulatrix and for longer periods (3 months or more) in a second storage organ, the seminal receptacle, which usually is located deep within the oviduct (Buckland-Nicks *et al.*, 1999). In other animals where multiple paternity occurs, this scenario of two sperm storage organs may be correlated with cryptic female choice, in which the female selects among the sperm of several rival males (see review by Birkhead and Møeller, 1998). Some gastropods are capable of using stored sperm for more than one year (Trüb, 1990; Baur, 1998). Furthermore, in pulmonates, one copulation per reproductive season is sufficient to fertilize all the eggs produced by an individual in one year, which suggests that

females control sperm release from the seminal receptacle, using only a fraction of stored sperm for each batch of eggs (Chen and Baur, 1993; Haase and Baur, 1995).

Molecular genetic markers have been used very effectively in the past to create DNA “fingerprints” that can genetically determine paternity. Previous studies of gastropod relationships have suggested that starch gel electrophoresis is inadequate to “elucidate the ecological dynamics of fertilization and paternity” (Gaffney and McGee, 1992), whereas the high variability at microsatellite DNA loci enables a more powerful analysis of parental relationships.

The intertidal snail *Littorina obtusata* is gonochoric. Females lay benthic egg masses containing 50–150 eggs (Bandel, 1974) in which embryos undergo direct development and emerge as crawling juveniles. In the present study, single locus microsatellite DNA markers were used to determine paternity of *L. obtusata* juveniles hatched from three egg masses kept in isolation in aquaria with several potential sires and the attendant dam in each case.

Materials and Methods

Samples

Adult specimens of *Littorina obtusata* were collected in early May 1999 from sites along the northeastern shore of Nova Scotia, Canada. Each shell was marked for gender and site number with nail varnish. All female snails used in the breeding experiment were collected from the same site (Port Bickerton) to ensure that all were in a relatively synchronous breeding cycle. Males were collected from five sites (Table 1) to increase the chance of genetic variability among the competing sires and thus increase the ability to assign paternity to the resulting offspring.

Individual females were isolated with 4–5 males and kept in 20-l aquaria at 8°C with a 12-h light/dark cycle. Mating

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Table 1

Collection sites in Nova Scotia of male *Littorina obtusata* used in breeding and paternity analysis

Location	Male ID code	Position
Port Bickerton	PB	45° 06'N, 61° 43'W
Fisherman's Harbour Wharf	FH	45° 07'N, 61° 40'W
Isaacs Harbour	IH	45° 12'N, 61° 39'W
Seal Harbour Wharf	SH	45° 09'N, 61° 34'W
Tor Bay Provincial Park	TB	45° 14'N, 61° 19'W

behavior was observed opportunistically throughout the experiment. Males that were observed in a stationary position, forward, and on the right side of the female's shell with the shell-lip and mantle touching, were recorded as "copulating." Males that were observed similarly positioned but slightly distal and without contact between the male mantle and female shell-lip were recorded as "possible mate-guarding" (see Discussion). Once egg masses were laid, the water temperature was increased to 10°C, which was reported to be a suitable temperature for egg development in the congener *Littorina sitkana* (Buckland-Nicks, 1974). Three additional males were included in the analyses for one brood (brood C). These males had escaped into the study tank and were then considered as potential sires.

When the juveniles reached about 1.4 mg in weight, they were frozen in a physiological buffer (10 mM Tris, pH 6.8) at -86°C. Adults were removed from their shells and frozen at -86°C in buffer following successful oviposition by the female.

DNA extraction

For each adult snail, a section of the foot was excised, rinsed with double-distilled H₂O and chopped, using a fresh razor blade, on a clean glass plate. This tissue was placed in 100 µl of TE (10 mM Tris-HCl pH 8, 1 mM EDTA) plus 0.5% Tween 20, and incubated at 60°C with 3 µl proteinase K (20 mg/ml). Following complete digestion, the DNA was isolated using a standard phenol:chloroform method (Sambrook et al., 1989). The DNA was further purified by

passing it through polyvinylpolypyrrolidone spin columns (Berthelet et al., 1996; Paterson and Snyder, 1999) to ensure the removal of compounds that are known to co-purify with molluscan DNA and inhibit the polymerase chain reaction (Winnepenninckx et al., 1993; Mikhailova and Johanneson, 1998). DNA concentration was estimated for each individual against Lambda HindIII molecular weight marker using agarose gel electrophoresis (Maniatis et al., 1982).

DNA from individual juveniles (~1.4 mg wet weight) was isolated using the following protocol. Each juvenile was rinsed with double-distilled H₂O and placed in an Eppendorf tube with 40 µl of TE plus 0.5% Tween 20, then crushed against the side of the tube with a micropipetter tip. Two microlitres of proteinase K (20 mg/ml) was added, and the tube was incubated at 60°C for 20 min, followed by a 5-min incubation at 95°C to denature the proteinase K. The tubes were spun briefly to pellet the shell debris. To avoid any loss of DNA during subsequent purification steps, no further purification was conducted.

DNA amplification

DNA was screened for microsatellite repeats at 12 loci using primers designed on the congener *Littorina subrotundata* (Tie et al., 2000). Of the 12 primer pairs, 8 did not produce scorable amplifications, and one locus was invariant (Lsub 6). Three loci were used for the analyses (Table 2). Approximately one-third of the offspring of each brood was typed at these three loci (40–46 juveniles per brood).

Amplifications were carried out by polymerase chain reaction (PCR) in 5-µl volumes containing ~0.2 ng DNA, 0.08 µM γ33P-ATP end-labeled forward primer, 0.42 µM forward primer, 0.5 µM reverse primer, 20 µM each dNTP, 0.2–0.5 units Taq polymerase, and 1× buffer containing 20 mM Tris pH 8, 50 mM KCl, 1.5 mM MgCl₂, and 0.1% Tween 20. Reactions were performed in an MJ Research PTC 100 thermocycler using an initial denaturation of 3 min at 94°C, followed by 25 cycles of denaturing at 93°C for 30 s per cycle, followed by optimal annealing (Lsub 8: 60°C; Lsub 32: stepdown from 55°C to 48°C; Lsub 62:

Table 2

Microsatellite loci used for paternity assessment in *Littorina obtusata*

Locus	Repeat motif*	PCR product size range (bp)	Number of alleles	Primer sequence (5'–3')
Lsub 8	(ATA) ₂₉	178–211	10	ccagtgaccagatcatagcg ggaattgtaagtgctggagc
Lsub 32	(CAA) ₁₃ TG(AAC) ₅ TG(AAC) ₁₀	210–343	14	atcacatgcacacgcttac acggtgtgtcatcaaacg
Lsub 62	(AAC) ₁₄	160–187	8	cgcttcccgtataccaac caccgtaaacctgtgagc

* From Tie et al. 2000.

64°C) for 40 s + 1 s per cycle. Products were separated using 6% denaturing polyacrylamide gel electrophoresis and visualized using autoradiography (Hillis *et al.*, 1996).

All individuals were scored at each locus except for one offspring of female A which did not amplify and was discarded, and two offspring of female B which were not typed at one locus each. Maternity was confirmed by direct comparison to the maternal genotype.

Statistical analyses

Paternity was initially assessed using exclusion, wherein putative fathers were excluded if there were any mismatches between their alleles and those of the offspring. Subsequently, paternity inference was conducted using CERVUS software (Marshall *et al.*, 1998; Slate *et al.*, 2000), which is a Windows-based maximum likelihood program designed for use with co-dominant markers. Paternity assignment using likelihood techniques was determined at an 80% confidence threshold. Marshall *et al.* (1998) suggested that 80% confidence in paternity is more accurate than can be achieved using direct observation, and better than can be achieved using exclusionary approaches because the degree of confidence in non-excluded males is unknown in the exclusion technique. Paternity is inferred from the log-likelihood ratios (LOD score) based on the genotypes of the offspring, candidate sire, and dam. Confidence levels are determined through simulation (10,000 iterations) and defined by the statistic delta (Δ) where Δ is the difference between the LOD scores of the two most likely candidates.

Input parameters for the CERVUS software have important effects on the resulting paternity assignments. The "error rate" parameter of the simulation program allows an estimate of scoring error to be included in the calculations. Allowing for error prevents exclusion of true paternal candidates due to errors causing single locus mismatches, while potentially including (erroneously) sires that would otherwise be excluded. As the number of loci sampled increases, this error estimator becomes more important due to the increased probability of mis-scoring or mutation (Slate *et al.*, 2000). Our data set is small and all scores were double-checked for accuracy, so the error rate is probably lower than the 1% adopted by Marshall *et al.* (1998). Another important simulation parameter for this data set is the proportion of males sampled. We assumed a minimum number of non-sampled males (9, see Discussion).

Results

Behavioral observations

Mating behavior in *Littorina obtusata* involves pursuit of a female by a male during which he crawls over the right side of her shell and inserts the penis, engorged with hemolymph, into her gonopore, which opens into the oviduct

and bursa copulatrix. A series of ventral mammiform penial glands are brought into contact with the shell and mantle during this process. Male ejaculate exits the prostate gland in a dorsal penial groove and passes along the groove with the aid of ciliary action. On several occasions when copulating pairs were separated, ejaculate was seen moving along the penial groove toward the tip of the penis.

A total of 19 copulatory pairings and 8 incidences of suspected mate-guarding were observed. Males remained in copulatory position with females for 1–4.5 h and occasionally remained on the female, without genital contact, for up to 2.5 h after copulation. Each female was observed copulating with more than one male, and repeat copulations by individual males occurred in a number of cases. Eggs were laid from 7 to 21 days after introduction of females to the aquaria.

Microsatellite analyses

Paternity of each brood involved not only sampled males from aquaria but also non-sampled males that had copulated with females prior to capture. Assessment of paternity through exclusion (putative parents are excluded as true parents if a mismatch occurs at any allele that is present in the offspring) suggested that a minimum of 2 of the sampled males contributed to each brood. Additionally, the presence and distribution of the alleles that were not contributed by the sampled males requires a minimum of 2 non-sampled sires of brood A, 4 non-sampled sires of brood B, and 3 non-sampled sires of brood C to explain the genotypes found in the offspring. Based on exclusion, these non-sampled males contributed from 22.5% (9 of 40, family C) to 70.7% (29 of 41, family A) of the offspring per brood. Thus, the alleles present in the offspring are accounted for by a minimum of 4 sires of brood A, a minimum of 6 sires of brood B, and a minimum of 5 sires of brood C.

Determination of paternity based solely on exclusionary methods is simplistic and, in this case, inadequate to accurately resolve paternity. Paternity assessment was therefore conducted using CERVUS software to enable paternity assignments with 80% confidence based on maximum likelihood calculations. An important aspect of the CERVUS likelihood analyses is the estimate of scoring error (Marshall *et al.*, 1998). We determined paternity assignment for a range of scoring-error estimates (from 0% to 1.0%); however, results were similar and only 0.5% assignments are presented here (Table 3). Paternity was assigned with 80% confidence in 19.5% of the offspring of brood A, 40.8% of brood B, and 20.0% of brood C. Paternity assignment was recalculated for each brood by using a modified data set that included the two most likely non-sampled male genotypes inferred from the genotypes of the non-assigned offspring. With inclusion of the inferred male genotypes,

Table 3

Summary of paternity inference at 80% confidence computed using the CERVUS program

Female	Candidate sire (number of obs. matings)	Known paternal genotypes		Including inferred paternal genotypes	
		Offspring sired	Percent sired	Offspring sired	Percent sired
A	PB1 (1)	2	4.9	2	4.9
	FH1 (1)	0	—	0	—
	IH1 (0)	0	—	0	—
	SH1 (0)	6	14.6	0	—
	TB1 (3)	0	0	3	7.3
	No assignation	33	80.5	6	14.6
	Unknown A1	n/a	n/a	25	61.0
	Unknown A2	n/a	n/a	5	12.2
B	PB2 (3)	11	23.9	17	37.0
	FH2 (2)	8	17.4	14	30.4
	SH2 (2)	0	0	0	0
	No assignation	27	58.7	8	17.4
	Unknown B1	n/a	n/a	2	4.3
	Unknown B2	n/a	n/a	5	10.9
	Total assigned	36	28.0	89	70.0
C	PB4 (2)	7	17.5	7	17.5
	FH4 (1)	0	0	1	2.5
	SH4 (0)	0	0	0	0
	TB4 (5)	0	0	0	0
	IH3 (0)	1	2.5	1	2.5
	SH3 (0)	0	0	0	0
	PB3 (0)	0	0	3	7.5
	No assignation	32	80.0	24	60.0
	Unknown C1	n/a	n/a	1	2.5
	Unknown C2	n/a	n/a	3	7.5
	Total assigned	36	28.0	89	70.0

Paternity assignment is indicated for a scoring-error estimate of 0.5%. Unknowns indicate the two most likely non-sampled sires inferred from the observed genotypes.

paternity was assigned for 85.4%, 82.6%, and 40.0% of the offspring in the three respective broods (Table 3).

Multiple mating was observed in all three females. Multiple paternity is clearly evident within the three broods, but the degree of paternal success varies throughout. Results produced using the modified data set (inferred male genotypes included) indicate that brood A was dominated by a single sire (61.0%, unknown A1); brood B had two relatively equally successful sires (37% for PB2, 30.4% for FH2) with at least four other sires contributing less than 11% each; and brood C was sired by at least five males, with no clearly dominant contributors.

Discussion

Duration of copulation and mate guarding

The duration of copulations we observed for *Littorina obtusata* in the laboratory varied from 1 to 4.5 h, which is in agreement with Ankel's (1936, cited in Baur, 1998) observations for this species and similar to times reported from some other prosobranchs (Martel *et al.*, 1986). In contrast, Struhsaker (1966) reported copulatory pairing to

last 30–45 min in *L. picta* and only 5–10 min in *L. pintado*. These differences in duration of mating may reflect real differences in reproductive behavior or morphology among different littorinids, including the presence or absence of penial glands, which are thought to secure the penis in place during copulation (Reid, 1989; Buckland-Nicks and Worthen, 1992). Parker (1970a) suggested that in some animal species, such prolonged copulations could be the male's way of reducing the probability of female remating by acting as a mechanical plug. Males of many animal species exhibit "passive phase behavior" in which they guard reproductive females after copulating (*e.g.*, Parker and Smith, 1975; Parker, 1984). The males in this study exhibited such behavior, remaining on the right side of the female's shell without genital contact for up to 2.5 h after copulation. An extended mating time plus guarding behavior may allow sufficient time for sperm to be transferred from the bursa copulatrix to the seminal receptacle, thereby increasing the male's chance of paternity. The time taken to transfer sperm between storage organs is not known for the Littorinidae, but Martel *et al.* (1986) reported that

sperm are transferred quickly from the bursa to the seminal receptacle in *Buccinum undatum*, taking at the most 3 days.

Multiple paternity

Multiple paternity has been demonstrated in a number of molluscan species. Among gastropods, studies of multiple paternity have been largely restricted to pulmonates (e.g., Mulvey and Vrijenhoek, 1981; Rollinson *et al.*, 1989; Wethington and Dillon, 1991; Baur, 1994), with fewer investigations conducted on marine prosobranchs (e.g., Gaffney and McGee, 1992). However, multiple copulations by females with different males have been reported in a wide range of gastropod families, suggesting that multiple paternity is common (see Martel *et al.*, 1986). Evidence of multiple paternity in *L. obtusata* was expected from both field and laboratory observations of multiple copulations by individual females. In this study, the number of males contributing to each brood is relatively high, with an absolute minimum of 4–6 sires per brood. This may be an underestimate of the number of true sires since only a fraction of each brood was tested, and a number of the alleles examined were fairly common.

The candidate sire that had immediate access to the females prior to spawning (*i.e.*, sampled males) sired fewer offspring (average of 37.8%) than the previously mated males (non-sampled), providing no evidence of last-male precedence. This is in agreement with a study of pseudoscorpions by Zeh and Zeh (1994), who found last-male precedence to be typical of two male systems (see also Parker, 1970b; Parker and Smith, 1975; Gwynne, 1984), but found no evidence of last-male precedence in broods from more promiscuous females.

The success rate of individual males appears highly variable—the dominant sire contributed from 61% (non-sampled A1, brood A) to 17.5% (PB4, brood C) of the offspring. Although the dominance of brood A by a single male is suggestive of first-male precedence, this is not upheld in the other broods. Indeed, brood B had greater than half of the offspring sired by “later” males (67.4%), and no previously mated males contributed to more than 11% of the offspring (Table 3). Thus, first-male precedence is not the prevalent reproductive mode in *L. obtusata*.

Copulation does not necessarily indicate successful sperm transfer to the female, because the male may not have inserted his penis or may not have delivered ejaculate (Gibson, 1964). However, when Buckland-Nicks *et al.* (1999) examined sperm transfer in the penis of *Littorina littorea* specimens that had been flash-frozen during confirmed copulation, they determined that sperm transfer often had been occurring at the time of disruption. In the present study, incidence of copulation was inferred based on male position and duration of coupling. Pairs invariably separated when

picked up and, as in observations made by Gibson (1964) on *L. planaxis*, ejaculate was observed moving along the penis in some instances. It is probable that sperm transfer was not occurring during some observations of “copulation”; for example, male TB4 was observed mating 5 times and male SH2 observed 2 times (Table 3), yet neither was successful in siring offspring.

In *L. obtusata* the high degree of multiple paternity in the resulting offspring indicates that sperm must be mixing within the female’s reproductive tract. Although release of sperm from the seminal receptacle may merely be the result of the female contracting the muscle layer surrounding the seminal receptacle (Buckland-Nicks and Chia, 1990), post-copulatory selection is also a possibility. This could occur either through sperm competition within the seminal receptacle or bursa copulatrix, or through post-copulatory female choice. Post-copulatory female choice may be accomplished in a number of ways, such as by dumping unwanted sperm, as observed in *L. scabra* (reported in Buckland-Nicks, 1998), by sperm digestion in the bursa copulatrix (see reviews by Buckland-Nicks, 1974; Fretter and Graham, 1994), or by sperm sorting and differential use within the reproductive tract (Haase and Baur, 1995).

On sperm storage organs

How does such short-term mate guarding increase the male’s chances of successful paternity? Species with a single blind-ended sperm storage organ usually show a pattern of last-male precedence in siring offspring, because rival males have the opportunity to actively disrupt or remove any sperm already stored there (e.g., Waage, 1979, 1984) or to passively displace the sperm deeper, rendering it ineffectual (e.g., Lefevre and Jonsson, 1962; Parker, 1970b). However, last-male precedence is not prevalent in species with two sperm storage organs, like littorinid snails, where there is an opportunity for the first male’s sperm to be transferred to the second storage organ before physical interference is possible by a subsequent male.

Besides the number of storage organs present, the shape of the sperm storage organs also has an effect on sperm precedence. Species which exhibit last-male precedence often have a relatively long, tubular, blind-ended storage organ, which is thought to promote stratification of sperm, with older sperm being overlaid by sperm from successive matings (e.g., Walker, 1980; Briskie and Montgomerie, 1993). However, Zeh and Zeh (1994) suggest that though tubular morphology may favor sperm stratification, this relationship breaks down if the storage organ becomes completely filled, which causes increased pressure and induces sperm mixing.

Spherical storage organs are thought to promote sperm mixing (Walker, 1980). In *L. obtusata*, the bursa copulatrix is the primary sperm storage organ. The bursa is a blind-

ended sac, providing the potential for sperm stored there to be disturbed by the penial filaments of rival males. Although there is no evidence of physical sperm displacement from the bursa copulatrix in *Littorina*, this remains a possibility because penial filaments are extensible and very mobile (Buckland-Nicks, 1974; Buckland-Nicks *et al.*, 1999). The secondary sperm storage organ, the seminal receptacle, is much smaller than the bursa and is situated deep within the pallial oviduct, beyond the reach of the penial filament (Buckland-Nicks and Chia, 1990). The longer a male guards a female, preventing her from participating in further mating, the greater the likelihood of his sperm leaving the bursa copulatrix and becoming stored in the seminal receptacle, to which male genitalia have no access.

Whether female littorinids can selectively store and use sperm from the seminal receptacle is not known. Our results for two of the three broods (A and C) indicate that the males that mated prior to capture had more success than the last males to mate, even though these later males were isolated with the female for up to 3 weeks. Moreover, males from places other than the home locale of the females (Port Bickerton) had little to no success. It is thus possible that females have some ability to stratify sperm in the seminal receptacle or to dump sperm from the bursa copulatrix. In this context, Haase and Baur (1995) suggested that females may be able to manipulate fertilization of eggs by storing sperm in different areas of the seminal receptacle. In *L. obtusata*, one might expect that the first sperm to reach the seminal receptacle would be stored close to its exit, favoring first-male precedence. However, our results support neither first nor last-sperm precedence and suggest there is mixing of sperm within the female's reproductive tract.

The results of paternity from the three egg masses of *L. obtusata* raise some intriguing questions about sperm selection. To fully understand the occurrence of sperm competition or cryptic female choice in these snails, experiments must be designed that track the sperm of individual males inside the female, as well as in the resulting offspring. This might be accomplished by using sperm-specific fluorophores, genetic markers, or the tried and tested method of radioautography (Beeman, 1970).

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